Lymphocyte Migration and Multiple Sclerosis: Relation with Disease Course and Therapy

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Lymphocyte migration into the central nervous system is a central event in lesion formation in multiple sclerosis. By using a fibronectin-coated membrane Boyden chamber assay, we observed that migration rates of immediately ex vivo lymphocytes from patients with relapsing–remitting, with or without concurrent clinical relapse, or with secondary progressive disease, were increased compared with healthy donors. Migration rates of lymphocytes from relapsing–remitting multiple sclerosis patients receiving either glatiramer acetate (Copaxone 20 mg daily) or interferon-β1b (Betaseron 8 MIU, three times per week) were significantly reduced compared with untreated relapsing–remitting patients. In vitro treatment with interferon-β1b (1,000 U/ml), but not glatiramer acetate (20 μg/ml), significantly reduced lymphocyte-migration rates, suggesting that the effects of these two therapeutic agents on migration result from different mechanisms of actions. Interferon-β1b acts, at least in part, by a direct effect on this cell property, whereas glatiramer acetate effects are indirect.

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Multiple sclerosis (MS) is characterized by development of multifocal lesions disseminated in time and space throughout the central nervous system. The typical initial relapsing–remitting (RR) phase of disease features a high frequency of magnetic resonance imaging (MRI)-defined gadolinium-enhancing lesions, an indication of disruption of the blood–brain barrier. The pathological substrate of such lesions is characterized by perivascular lymphocyte accumulation with extension into the parenchyma.[1,2] The sequence of cellular events whereby T cells access the central nervous system includes an active transmigration process that is dependent, at least in part, on production by the lymphocytes of proteinases, including matrix metalloproteinases (MMPs), which degrade the thin but compact basal lamina surrounding the brain microvessels.[3–6] An estimated 50% of MS cases evolve into a secondary progressive phase with or without intermixed relapses. In these cases, gadolinium-enhanced MRI lesions continue to occur.[7]

We have previously used a Boyden chamber containing a fibronectin-coated membrane as an in vitro model of lymphocyte migration.[8] In this model, migration reflects both the ability of T cells to bind to fibronectin and to degrade it, by an MMP-dependent proteolytic mechanism. We observed that interferon-β1b (IFNβ1b; Betaseron), added in vitro to activated lymphocytes, inhibited both migration rate and MMP production by these cells. We further found that immediately ex vivo peripheral blood–derived lymphocytes obtained from RR MS patients, without concurrent clinical relapse, had increased rates of migration in this assay when compared with controls.[9] Patients receiving IFNβ1b therapy had reduced migration rates compared with untreated patients.

The purpose of the current study was to evaluate migration of lymphocytes derived from MS patients in relation to both their disease phase (RR with and without active relapses or secondary progressive [SP]) and therapy being received (IFNβ1b and glatiramer acetate [GA; Copaxone]). We found that migration rates were increased in all untreated patient groups compared with healthy controls. Migration rates of lymphocytes from patients treated with either agent were reduced compared with untreated patients. In contrast, IFNβ but not GA, when added in vitro, reduced lymphocyte-migration rates.

Patients and Methods

Patients

The immediately ex vivo lymphocyte studies were conducted on the groups of clinically definite MS patients listed in the Table.[10] Most of the untreated RR patients were those with relatively recent disease onset (mean, 48 ± 7 months) who fulfilled local criteria to be eligible for the currently approved MS therapies (two relapses within the previous 2 years) and who were about to begin therapy. Active relapse was defined according to criteria used in clinical trials with IFNβ and GA. Blood was drawn from MS active relapse patients before initiation of steroid therapy. None of the SP patients were on immunosuppressive therapy. As expected, the SP patients were significantly older (mean, 47 ± 6 years) and had a longer disease duration than the RR patient groups (mean, 82 ± 9 months).

Lymphocyte Preparation

For the immediately ex vivo studies, mononuclear cells (MNCs) were isolated from peripheral blood samples fr m

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Table. Mean Age, Disease Duration, Expanded Disability Status Scale Score (EDSS), and Treatment Duration

Subgroups	n	Age (yr)	Disease Duration (mo)	EDSS	Treatment Duration (mo)
MS RR	7	39 ± 4	47 ± 7	1.9 ± 0.3	0
MS AR	12	35 ± 3	46 ± 12	1.7 ± 0.4	0
MS SP	9	47 ± 6	81 ± 9	2.9 ± 0.5	0
MS Betaseron	7	37 ± 3	34 ± 4	2.2 ± 0.2	24 ± 4
MS Copaxone	10	36 ± 6	39 ± 5	2 ± 0.4	6 ± 3
Controls	14	30 ± 2	0	0	0

Patient subgroups are those with stable relapsing–remitting disease (RR), active relapse (AR), and secondary progressive (SP) disease. Treated patient groups were those with relapsing–remitting disease receiving either interferon-β1b (Betaseron) or glatiramer acetate (GA; Copaxone).

patients or controls by using a Ficoll density gradient (Pharmacia Biotech, Baie D'Urfee, Quebec, Canada), depleted of monocytes by a 1-hour culture at 37°C in RPMI media plus 10% fetal bovine serum (FBS; Medicorp, Montreal, Quebec, Canada) in a 75-cm^2 plastic dish. Subsequently, T cells were suspended at 10^6 cells/ml in culture medium composed of RPMI plus 2.5% FBS and then used in the migration assay as described below. Anti-CD3 antibody staining and fluorescence-activated cell sorting (FACS) analysis revealed that over 95% of the cells were $CD3^+$ lymphocytes.

For the in vitro studies, MNCs were isolated from healthy donors, suspended in 10 ml of RPMI plus 10% FBS at 10^6 cells/ml and cultured in 75-ml flasks (Falcon-VWR, Montreal, Quebec, Canada) for 72 hours in the presence or absence of either IFNβ1b (1,000 U/ml) or GA (20 μg/ml). The IFNβ dosage used was based on previous dose–response studies.[8] The GA dosage was the one that we have found to induce optimal generation of GA-reactive T-cell lines. After 72 hours, the nonadherent cultured cells were harvested and resuspended in fresh RPMI plus 2.5% FBS for use in the migration assay; 10^5 cells were also pulsed with [^{3}H]thymidine in flat-bottomed 96-well plates for 5 hours, and proliferation index was assessed by harvesting the cells and counting on a Beckman β-counter (Fisher Scientific, Montreal, QB, Canada).

Migration Assay

All assays were conducted in Boyden chambers (3-μm pore size membranes) precoated with fibronectin (Collaborative Biomedical Products, Bedford, MA). The bottom chamber contained 1 ml of RPMI plus 10% FBS; 10^6 lymphocytes suspended in 1 ml of RPMI plus 2.5% FBS was added to the top chamber. After 6 hours at 37°C, the contents of the bottom chamber were collected and the number of cells present determined by counting aliquots under the microscope.

Statistics

For each donor, lymphocyte migration was performed in duplicate and mean values determined. Results are presented as mean ± SEM values of the number of donors studied in each subgroup. Statistical comparison between the groups was performed by using an analysis of variance test and a Dunnett post test.

Results

Immediately Ex Vivo Studies

As shown in Figure 1, the migration rates of T cells derived from untreated MS patients, either RR or SP, were significantly higher than were the rates of migration of cells derived from healthy control donors ($p < 0.01$). There was no significant difference in migration rates between RR patients who were or were not actively relapsing. The migration rates of lymphocytes derived from both IFNβ- and GA-treated patients was significantly reduced compared with the untreated RR patients ($p < 0.05$) and not different from migration rates of control donors.

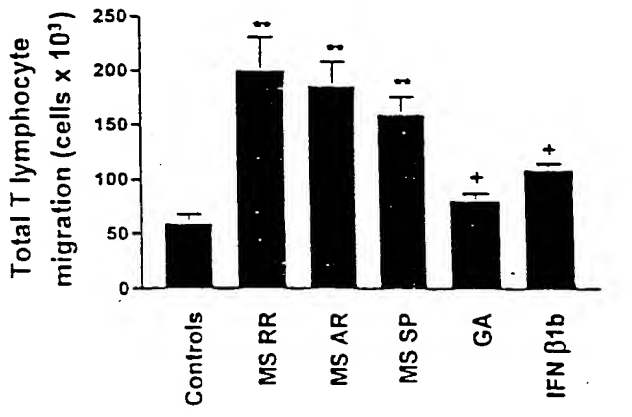

*Fig 1. Migration rates of immediately ex vivo lymphocytes derived from peripheral blood of controls, untreated relapsing–remitting multiple sclerosis (MS) patients with stable disease (RR) or active relapse (AR), secondary progressive MS patients (SP), or relapsing–remitting patients treated with interferon-β1b (IFNβ1b) or glatiramer acetate (GA). Data indicate mean number of cells ± SEM values from the lower chamber of the recovered Boyden chamber after 6 hours. (**p < 0.01: MS RR, MS AR, or MS SP, compared with control; *p < 0.05: GA or IFNβ1b, compared with nontreated MS RR).*

In Vitro Studies

The lymphocytes maintained in culture media alone for 3 days had significantly higher migration rates than did immediately ex vivo cells (3.12×10^5 compared with 5.9×10^4, respectively; $p < 0.01$). As shown in Figure 2, the numbers of migrating cells derived from cultures to which IFNβ had been added were significantly reduced compared with those derived from control cultures containing media alone ($p < 0.01$). In contrast, migration rates of cells derived from GA-treated cultures did not differ from controls. [^{3}H]Thymidine uptake by GA-treated cells (3,155 ± 607, n = 5) was significantly increased ($p < 0.05$) compared with nontreated cells (1,166 ± 357, n = 6); there was no significant difference in proliferation rate between cells cultured with IFNβ (2,014 ± 318, n = 5) and GA.

Discussion

In the current study, although we confirmed our results that migration rates of immediately ex vivo peripheral blood–derived lymphocytes were increased in the stable RR subgroup,[9] we could not distinguish stable and relapsing patients. MRI-based studies have repeatedly demonstrated recurrent new lesion formation in RR patients even without clinical counterparts. The lymphocyte-migration rates in the SP MS patient group were comparable with those of the RR group. SP patients do continue to develop new MRI-defined lesions.[7] Levels of MMP-9 are increased in the CSF of both RR and progressive MS patients.[11] In contrast to our migration results, SP and RR patients do show significant differences in several cell-mediated immune functions including production of the proinflammatory cytokines IFNγ, tumor necrosis factor-α, and interleukin-12.[12–14] These findings suggest that the mechanisms underlying lymphocyte migration may be dissociated from other functional immune responses considered to contribute to the MS disease process.

In this study, we have compared the in vivo and in vitro effects of IFNβ1b and GA on the rate of lymphocyte migration. Migration rates of immediately ex vivo lymphocytes obtained from IFNβ- or GA-treated patients were significantly reduced compared with untreated patients, whereas only IFNβ exerted a significant effect when added in vitro. For the in vitro drug effect studies, we elected to use control donor-derived MNCs cultured for 3 days under basal conditions because the low migration rate of these donors' MNCs immediately ex vivo precluded detection of a further decrease. IFNβ effects on clinical and MRI-defined disease activity are apparent from a very early time point after initiation of therapy.[15] This would seem consistent with our in vitro findings, showing a direct effect of IFNβ on lymphocyte migration. The apparent dissociation between the in vivo and in vitro lymphocyte-migration data, from using GA, suggests the in vivo effects reflect an indirect effect on this lymphocyte property. Whether such an indirect effect reflects a specific regulatory mechanism, or an overall reduction in state of lymphocyte activation, remains speculative. Our conclusion regarding an indirect in vivo effect of GA on lymphocyte migration would seem consistent with recent observations that the effects on MRI activity become more apparent after several months of therapy.[16] We speculate whether the lymphocyte-migration assay may serve as an overall index of immunologically related disease activity in a manner parallel to that used by disability scales to provide an index of disease severity. Prospective studies are needed to determine whether persistently high migration rates will correlate with treatment failure, using currently available agents. The measure of migration may also be well suited for studies, assessing the potential of combination immunomodulatory therapies[17] involving agents that act via different mechanisms.

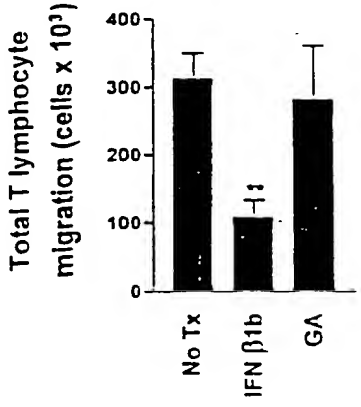

*Fig 2. Migration rates of lymphocytes cultured for 72 hours under basal conditions and placed in Boyden chamber without treatment (no Tx) or in presence of interferon-β1b (IFNβ1b) or glatiramer acetate (GA). Data indicate mean number of cells ± SEM values recovered from the lower chamber of the Boyden chamber after 6 hours, in six experiments performed. (**p < 0.01: in vitro IFNβ1b-treated cells compared with nontreated cells).*

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DOCUMENT NUMBER: 132:6285
TITLE: Evaluation of chitosan used as an excipient in tablet formulations
AUTHOR(S): Kepsutlu, A. Riza; Savaser, Ayhan; Ozkan, Yalcin; Dikmen, Necati; Isimer, Askin
CORPORATE SOURCE: Department of Pharmaceutical Technology, Gulhane Military Medical Academy, Ankara, 06018, Turk.
SOURCE: Acta Pol. Pharm. (1999), 56(3), 227-235
CODEN: APPHAX; ISSN: 0001-6837
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LANGUAGE: English

AB Chitosan was chosen as a model excipient because it has many different functions in pharmaceuticals. This study was intended to investigate the use and optimum concn. of chitosan as a tablet binder and disintegrant. Chitosan was prepd. by 2 tabletting methods. In these methods, piroxicam was selected as an active substance. Tablet formulations were prepd. by granulating 3 viscosity grades of CMC (CM-cellulose) and 3 viscosity grades of PVP [poly(N-vinylpyrrolidone)] in varying ratios. Chitosan was evaluated as a binder for piroxicam tablets and compared with other polymer binders such as PVP. Again, a chitosan was evaluated as a disintegrant for piroxicam tablets as compared with other cellulose disintegrants such as CMC. In the wet method, chitosan decreased the release of the drug. Therefore, chitosan is considered to be useful as excipient for controlled release drug formulations. Chitosan is a pharmaceutical excipient of natural origin that may combine the binding and disintegrant properties.

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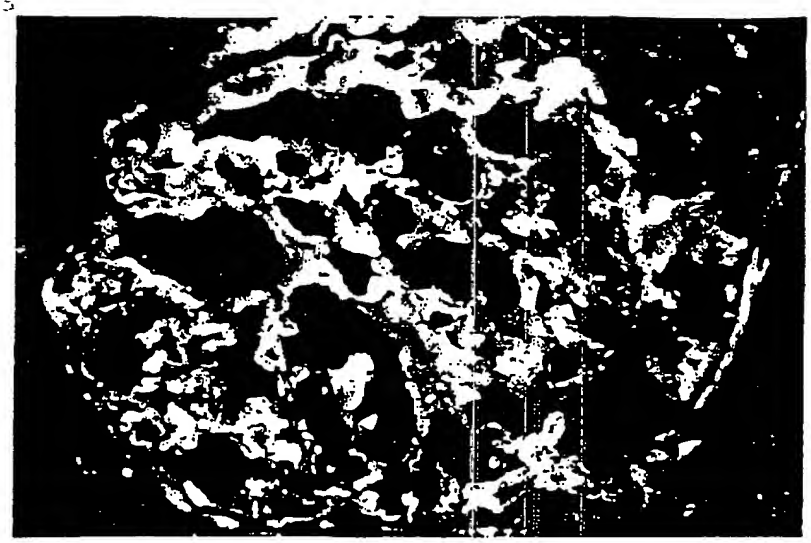

FIG. 11. Type III (immune-complex) injury in an SLE renal biopsy specimen. This patient had proteinuria and red blood cells in her urine. Note the granular (sometimes called "lumpy-bumpy") distribution of the immune deposits in this section stained with antibody to human C3.

early spontaneous abortion and may be an important cause of thrombotic disease in the general population.

Tissue damage may also be mediated through antibodies to neutrophil cytoplasmic antigens (ANCAs). These IgG antibodies, initially detected by immunofluorescence, have been divided by staining patterns into perinuclear (p-ANCA) and cytoplasmic (c-ANCA). p-ANCAs are directed against myeloperoxidase, while c-ANCAs are specific for proteinase 3 (221). These autoantibodies are useful markers for vasculitis, including Wegener's granulomatosis, pauciimmune necrotizing and crescentic pauciimmune glomerulonephritis, and polyarteritis nodosa, and their titers correlate with disease severity. The mechanism by which antibodies to these cytoplasmic antigens leads to blood vessel damage and inflammation is incompletely understood, but it may involve expression on activated neutrophils of proteinase 3 and myeloperoxidase, and possibly release of free proteinase 3. Antibodies to these molecules may provoke enhanced neutrophil chemotaxis and adhesion, together with triggering of the respiratory burst. This may lead to a series of events culminating in activation of T cells and macrophages and the formation of necrotizing granulomas.

APPROACHES TO THE TREATMENT OF SYSTEMIC AUTOIMMUNE DISEASE

In general, the management of human systemic autoimmune disease is empirical and unsatisfactory. For the most part, broadly immunosuppressive drugs, such as corticosteroids, are used in a wide variety of severe autoimmune and inflammatory disorders; in milder conditions, antiinflammatory agents acting on eicosenoid metabolism are often sufficient.

In addition to corticosteriods, other immunosuppressive agents are used in management of the systemic autoimmune diseases. Cyclophosphamide is an alkylating agent that causes profound depletion of both T- and B-lymphocytes and impairment of cell-mediated immunity. It is used in SLE nephritis and is particularly effective in granulomatous vasculitis and polyarteritis nodosa. Its use entails the risks of immunosuppression, along with an increased incidence of lymphoreticular malignancies. Azathioprine and the closely related 6-mercaptopurine are used in parallel situations; these are somewhat less effective but are less toxic.

Cyclosporine, tacrolimus, and mycophenolate mofetil are natural products with specific properties of T-lymphocyte suppression, and they have been used with success in SLE, RA, and, to a limited extent, in vasculitis and myositis. They have significant renal toxicity in addition to their immunosuppressive effects.

Methotrexate is widely used as a "second-line" agent in RA, with the goal of reducing disease progression. It is also useful in polymyositis and other connective-tissue diseases. Its mechanism of action here is controversial and may relate to its action on adenosine receptors rather than to its more familiar role as an antimetabolite.

There is optimism that more specific treatment for autoimmune disorders can be devised when their mechanisms become better understood. Oral tolerance holds promise as a means of attracting immunosuppressive T-lymphocytes to sites of active autoimmune pathology and suppressing inflammation by a bystander effect, probably involving TGF-β (271). Other approaches under development are monoclonal antibodies that are intended to block the action of cytokines or to deplete lymphocytes (204). With the exception of anti–TNF-α in RA (205), these have been disappointing.

CONCLUSIONS

The mechanisms of systemic autoimmune disease are diverse and incompletely understood. Several points are worthy of emphasis. The rules and restrictions governing ordinary immune responses seem to apply to autoimmune responses: there is little that is extraordinary about the immunoglobulin or TCR genes used or in their means of rearrangement or diversification; antigen is required to initiate responses. Production of and response to cytokines and other mediators is similar to what is seen for responses to exogenous antigens, and T and B cells collaborate in an MHC-restricted fashion. The availability of transgenic and knock-out mice and continuing progress in the understanding of the genome seem likely to open novel and fruitful approaches to understanding disorders of systemic autoimmunity.

Applicants: Alexander Gad et al.
Serial No.: 09/816,989
Filed: March 23, 2001
Exhibit 17

L47 ANSWER 3 OF 5 MEDLINE
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DOCUMENT NUMBER: 20060325
TITLE: [Treatment of multiple sclerosis. The present and the future. Study Group on Diagnosis and Therapy of Multiple Sclerosis].
Il trattamento della sclerosi multipla. Il presente ed il futuro. Gruppo di Studio per la Diagnosi e Terapia della Polisclerosi.
AUTHOR: Cazzato G; Antonello R M; Zorzon M; Torre P; Zivadinov R; Moretti R; Bragadin L M; De Masi R; Nasuelli D
CORPORATE SOURCE: Istituto di Clinica Neurologica, Universit`a, Trieste.. ncarraro@fmc.univ.trieste.it
SOURCE: RECENTI PROGRESSI IN MEDICINA, (1999 Oct) 90 (10) 538-44. Ref: 40
Journal code: R1T. ISSN: 0034-1193.
PUB. COUNTRY: Italy
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General Review; (REVIEW)
(REVIEW, TUTORIAL)
LANGUAGE: Italian
ENTRY MONTH: 200003
ENTRY WEEK: 20000302

AB The last years have produced a plethora of new information including extensive studies, retrospective analysis and new perspectives on data interpretation on multiple sclerosis (MS) treatment. Considering how difficult it is to study a disease such MS with its variability, unpredictability and duration, it seems hard to resemble definite results from this experience. However, corticosteroids have been the mainstay of treatment for the management of acute relapses, showing the capacity to shorten the duration of relapses, accelerate the recovery. At present, interferon beta is generally considered to be the treatment of choice for patients with relapsing remitting disease. **Glatiramer** acetate is still not available in many parts of Europe, but its results demonstrate a reduction of relapses in 30% of cases. Most European experts only consider as alternative treatment the immunosuppressive drugs, chosen if patients demonstrate unacceptable side effects of interferon or clearly do not respond. Very different and even more confusing data still come from experimental trial in secondary progressive MS, where the target of treatment is to slow the progression of disability. Different drugs (methotrexate, mitoxantrone, linomide, steroids and even interferons) are employed, but the results are still debated. Future therapies are being derived from constantly changing and evolving concept of MS immunopathogenesis: therefore many experimental and clinical trials use anti-integrin antibodies or insulin growth factors, metallo-**proteinase inhibitors** or T-cell vaccination. Some of the above treatment may have a chance of producing the gaining control of the disease without much inner toxicity.

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ACCESSION NUMBER: 2000:455053 HCAPLUS
DOCUMENT NUMBER: 134:114464
TITLE: Synthetic peptides that inhibit binding of the collagen type II 261-273 epitope to rheumatoid **arthritis**-associated HLA-DR1 and -DR4 molecules and collagen-specific T-cell responses
AUTHOR(S): Fridkis-Hareli, M.; Rosloniec, E. F.; Fugger, L.; Strominger, J. L.
CORPORATE SOURCE: Department of Clinical Immunology, Aarhus University Hospital, Aarhus, Den.
SOURCE: Hum. Immunol. (2000), 61(7), 640-650
CODEN: HUIMDQ; ISSN: 0198-8859
PUBLISHER: Elsevier Science Inc.
DOCUMENT TYPE: Journal
LANGUAGE: English

AB Copolymer 1 [**Cop 1**, poly (Y, E, A, K)] is a random synthetic amino acid copolymer effective in the treatment of relapsing forms of multiple sclerosis (MS), a disease that is linked to HLA-DR2 (DRB1*1501). Another copolymer [poly (Y, A, K)] was also identified that binds to rheumatoid **arthritis** (RA)-assocd. HLA-DR1 (DRB1*0101) or HLA-DR4 (DRB1*0401) mols. and inhibits the response of HLA-DR1- and -DR4-restricted T cell clones to an immunodominant epitope of collagen type II (CII) 261-273 (a candidate autoantigen in RA). In the present study various peptides have been synthesized based on binding "motifs" of **Cop 1** for HLA-DR1 and -DR4 mols. Those peptides with K at P-1 or K at P8 were particularly effective as inhibitors of binding of CII 261-273, of **Cop 1** and of the influenza virus hemagglutinin peptide 306-318 to these class II proteins. Moreover, several of them were also potent inhibitors of the CII 261-273-reactive T cell clones. These findings suggest that small peptides or their more stable derivs. may be able to substitute for copolymers in the treatment of RA, and by implication of MS.

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